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Methods for the Isolation and Characterization of Constituents of Natural Products

III. Separation of Alcohol Esters of Pyruvic Acid 2,6-Dinitrophenylhydrazone into Classes by Column and Thin-Layer Chromatography

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In the initial report of this series (2) methods were described for the preparation of a new acid chloride, namely, pyruvyl chloride 2,6-dinitrophenylhydrazone and esters prepared from it with primary, secondary, and tertiary aliphatic alcohols. Part II described normal- and reversed-phase partition systems for separating homologous series of members of the classes of alcohols by thin-layer chromatography (TLC) (3). The present report is concerned with the quantitative separation of a mixture of the ester derivatives into classes (namely, those derived from primary, secondary, and tertiary saturated alcohols) by column chromatography on magnesium oxide. The column procedure has been adapted qualitatively to TLC, and this technique is also described. The use of TLC on aluminum oxide to separate isomeric ester derivatives is also presented.

APPARATUS AND MATERIALS¹

Magnesium oxide (catalog no. 2477) suitable for chromatographic use was obtained from the J. T. Baker Co., Phillipsburg, N.J. The powder had an adsorption index (Food and Drug yellow No. 4) of 12-13 and was used without further treatment. Celite 545 and Analytical Grade Celite were obtained from the Johns-Manville Co., Baltimore, Maryland. Chloroform, benzene, and methanol were ACS grade; diethylamine (Baker) was

¹ Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

redistilled; methyl cyclohexane was obtained from Distillation Industries, Rochester, N.Y., Aluminum oxide G was obtained from Brinkmann Instruments, Inc., Westbury, N.Y.

The thin-layer chromatography equipment was the same as previously described (3). A borosilicate glass column $15.5 \times 1\frac{3}{8}$ inches I.D. containing a coarse fritted glass disc was employed for the column chromatography.

EXPERIMENTAL

Separation of classes by column chromatography. Magnesium oxide (7.5 g) and Celite 545 (30 g) are slurried together in approximately 90 ml of CHCl_3 . The slurry is poured through a long-stemmed funnel into the chromatography tube and packed immediately by moderate air pressure until a few milliliters of solvent remain above the bed. The esters, dissolved in the minimum amount of CHCl_3 , are quantitatively transferred to the column taking care not to disturb the top of the bed. After all of the solution has percolated into the bed, the sides of the column are washed down with two 1-ml portions of CHCl_3 , and when these have penetrated a plug of glass wool is placed just above the bed and development of the chromatogram is begun. The esters, adsorbed as a deep violet or bluish-violet band at the top of the column, are separated using the following sequence of solvents: 50 ml CHCl_3 , 100 ml of 0.1% MeOH in CHCl_3 , 100 ml of 0.2% MeOH in CHCl_3 , 100 ml of 0.5% MeOH in CHCl_3 , 50 ml of 2% MeOH in CHCl_3 and, finally, 5% MeOH in CHCl_3 until the last band is removed from the column.

Separation of classes by TLC. Magnesium oxide (3 g) and Analytical Grade Celite (7 g) are slurried in 50 ml of distilled water and spread in the usual manner over five 8×8 -inch plates. The plates are dried at 100°C for at least 2 hours. The esters are spotted from benzene solution and turn violet or blue as the benzene evaporates. The plate is developed in an equilibrated tank using benzene: CHCl_3 (3:1) as solvent. At the end of approximately 1 hour, the plate is removed and the spots marked as the solvent evaporates.

Separation of isomeric alcohol derivatives by TLC on alumina. Aluminum oxide G (25 g) is shaken with 35 ml of distilled water and the slurry is spread over four 8×10 -inch plates. The plates are activated at 100°C for at least 2 hours, then spotted with benzene solutions of the derivatives and developed in an equilibrated tank with diethylamine:methyl cyclohexane (35:65). At the end of the development (about 1 hour) the plate

is removed and the purple spots are marked. As the diethylamine evaporates from the plate, the purple spots revert to their original yellow color.

RESULTS AND DISCUSSION

Figure 1 depicts the type of separation of the classes achieved in the system described for column chromatography. The effluent from the column was monitored continuously at 400 m μ by running it through a 0.2-ml flow-through cell in an Hitachi-Perkin Elmer model 139 spectrophotometer and recording the readings with a Honeywell-Brown 5-mv recorder. For the attempted separation of the classes, the extremes in each class (of the derivatives prepared in this laboratory) were selected. Thus the C₈ and C₄ tertiary, the C₁₈ and C₃ secondary and the C₁₈ and C₁ primary alcohol derivatives were employed. The extremes were selected because preliminary work indicated that the most difficult resolution would be between a short-chain compound in the faster-moving class and a long-chain compound in the class immediately following it on the column. This is also the case in the separation of 2,4-dinitrophenylhydrazones into classes (4). The mixture selected also ostensibly represents a more difficult situation than will occur in an unknown consisting solely of saturated aliphatic alcohol derivatives.

All of the members lying between the extremes in chain length in a given class move the same or between the extremes in that class on the chromatogram. The tertiary alcohol derivatives move fastest, and primary alcohol derivatives slowest, on the chromatogram. All tertiary alcohol derivatives above tert-butanol move as blue bands on the adsorbent. The tert-butanol is violet as are all of the primary and secondary alcohol derivatives which have been prepared thus far.

The chromatogram shown in Fig. 1 took 3 hours to complete. However, the time to complete the separation in practice can be shortened considerably since the primary alcohols can be eluted as one band, if desired, once the last of the secondary alcohols has been eluted.

Only two lots of the magnesium oxide were available for evaluation. Both lots gave highly satisfactory separations, but the chromatograms were not identical. Each band was checked for purity and was identified by thin-layer partition chromatography as described in Part II of this series (3).

Stability and recovery of the esters were checked by running each compound on individual columns. Recoveries ranged from 95% to 104% when

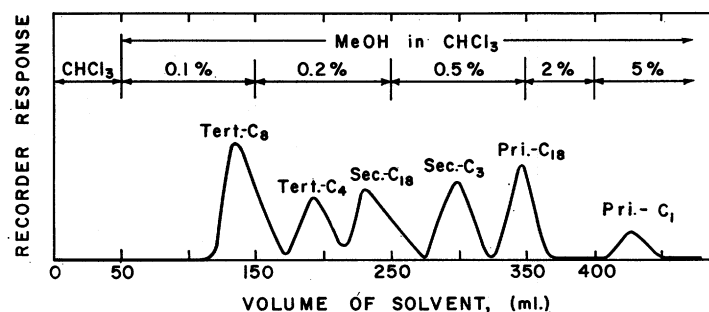


FIG. 1. Separation of a mixture of primary (pri), secondary (sec), and tertiary (tert) alcohol esters of pyruvic acid 2,6-dinitrophenylhydrazone into classes by column chromatography on magnesium oxide.

0.5 to 1.0 μ mole quantities were run. Similar amounts of esters were used in the mixtures. A 0.5 μ mole of an ester can be readily seen on the column, although the intensity of the band decreases as the band nears the bottom of the column.

Figure 2 shows the separation of the classes by TLC on a magnesium oxide plate. Separation is very good and the colors displayed on the magnesium oxide column are also manifested on the plate. Both lots of adsorbent gave equally good, but not identical separations. The same mixture which was chromatographed on the column was run on the plate, but, in addition, 2-butanol, *n*-propanol, and ethanol derivatives were also included. Approximately 5×10^{-4} μ mole of a derivative is still visible on the plate.

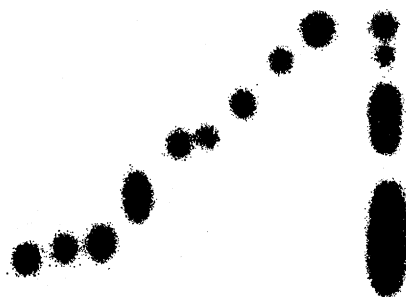


FIG. 2. Separation of a mixture of primary, secondary, and tertiary alcohol esters of pyruvic acid 2,6-dinitrophenylhydrazone into classes by TLC on magnesium oxide. Solvent, benzene: CHCl_3 (3:1). Diagonally from top to bottom: tert C_8 , tert C_4 , tert C_5 , sec C_{18} , sec C_4 , sec C_3 , pri C_{18} , pri C_2 , pri C_1 . Column on right represents a mixture of all of the compounds.

Separation is best achieved when approximately equimolar amounts of the derivatives are spotted. However, when there is a gross disproportionality of constituents, class separation is not as clear-cut and some confusion might arise concerning the proper classification of a derivative. In view of this shortcoming, it is recommended that class separation by TLC be used mainly as a check on the identity of a band obtained from column chromatograms since one can then spot on the plate approximately equimolar amounts of authentic derivatives and the unknown band.

It is also recommended that the prepared magnesium oxide plates be kept a maximum of 48 hours since there appears to be a weakening of the adsorbent when at oven temperature or at room temperature in a desiccator for longer periods.

Figure 3 shows the separation of isomeric alcohol derivatives. All primary and secondary alcohols from C_3 through C_{18} and the C_4 through the C_8 tertiary alcohols were examined. All isomeric alcohol derivatives separated in a similar fashion as the derivatives shown in Fig. 3. Under non-basic or even weakly basic conditions, isomer separation is not accomplished. Only strong bases with a pK_b less than 3.0 were effective. Isomer separation on a neutral or acidic (silica gel G works equally as well as aluminum oxide) adsorbent by using a strongly basic solvent system is probably comparable to the class separation described above on magnesium oxide. However, in attempting the class separation on aluminum oxide G or on silica gel G, no clean-cut separation was achieved. The short-chain components of the faster-moving class and the long-chain constituents of the class immediately following overlapped considerably and precluded the use of this system for executing separation of the classes. There is also no difference between the color of the spots of tertiary alcohols and that of the other alcohols, all spots being deep purple.



FIG. 3. Separation of isomeric alcohol esters of pyruvic acid 2,6-dinitrophenylhydrazone on aluminum oxide G. Solvent, methyl cyclohexane:diethylamine (65:35). (A) Top to bottom: tert C_6 , sec C_6 , pri C_6 ; (B) pri C_6 ; (C) sec C_6 ; (D) tert C_6 ; (E) Top to bottom: tert C_7 , sec C_7 , pri C_7 ; (F) pri C_7 ; (G) sec C_7 ; (H) tert C_7 ; (I) Top to bottom: tert C_8 , sec C_8 , pri C_8 ; (J) pri C_8 ; (K) sec C_8 ; (L) tert C_8 .

Separation of the 2,6-dinitrophenylhydrazone derivative of the pyruvate esters of primary, secondary, and tertiary alcohols into classes constitutes an important step in simplifying the analysis of complex mixtures of alcohols such as might be encountered in the analysis of a natural product. The value of having a method for class separation is exemplified by the progress made in carbonyl analysis in which the 2,4-dinitrophenylhydrazones are separated into classes (1, 4, 5).

SUMMARY

Quantitative column and qualitative thin-layer chromatographic procedures are described for separating a mixture of the 2,6-dinitrophenylhydrazone derivatives of pyruvic acid esters of primary, secondary, and tertiary aliphatic alcohols into classes. Magnesium oxide is used as the adsorbent in both procedures and separation of the classes follows a similar pattern. The derivatives change color from yellow to violet or blue on the adsorbent. Tertiary alcohol derivatives above butyl show a blue color whereas tertiary butyl and the primary and secondary alcohol derivatives are violet. Isomeric derivatives can also be separated by thin-layer chromatography on aluminum oxide G by using a solvent system containing a strong organic base.

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